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MINIREVIEW

pH Signaling in Human Fungal Pathogens: a New Target for Antifungal Strategies

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Fungi are exposed to broadly fluctuating environmental conditions, to which adaptation is crucial for their survival. An ability to respond to a wide pH range, in particular, allows them to cope with rapid changes in their extracellular settings. PacC/Rim signaling elicits the primary pH response in both model and pathogenic fungi and has been studied in multiple fungal species. In the predominant human pathogenic fungi, namely, *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*, this pathway is required for many functions associated with pathogenesis and virulence. Aspects of this pathway are fungus specific and do not exist in mammalian cells. In this review, we highlight recent advances in our understanding of PacC/Rim-mediated functions and discuss the growing interest in this cascade and its factors as potential drug targets for antifungal strategies. We focus on both conserved and distinctive features in model and pathogenic fungi, highlighting the specificities of PacC/Rim signaling in *C. albicans*, *A. fumigatus*, and *C. neoformans*. We consider the role of this pathway in fungal virulence, including modulation of the host immune response. Finally, as now recognized for other signaling cascades, we highlight the role of pH in adaptation to antifungal drug pressure. By acting on the PacC/Rim pathway, it may therefore be possible (i) to ensure fungal specificity and to limit the side effects of drugs, (ii) to ensure broad-spectrum efficacy, (iii) to attenuate fungal virulence, (iv) to obtain additive or synergistic effects with existing antifungal drugs through tolerance inhibition, and (v) to slow the emergence of resistant mutants.

Opportunistic fungal infections have emerged as a major cause of morbidity and mortality in immunocompromised patients, including those with AIDS, hematological malignancies, and stem cell and organ transplant recipients. Collectively, invasive candidiasis, cryptococcal meningitis, invasive aspergillosis, and pneumocystis pneumonia are estimated to cause at least as many deaths worldwide as tuberculosis or malaria (1, 2). However the treatment of these life-threatening infections caused by eukaryotic pathogens still lags far behind that of infections due to other microorganisms, such as bacteria. Only four classes of drugs are currently available for treating invasive fungal infections: polyenes, azoles, pyrimidines, and echinocandins (1). This limited arsenal of effective drugs highlights the urgent need for the development of novel therapeutic agents, particularly in light of the emergence of drug-resistant strains, the growing number of immunocompromised patients to be treated, and the toxicity, high cost, and narrow activity spectra of the drugs currently available (1, 3, 4). Two nonexclusive approaches may be considered in the field of antifungal drug discovery: finding new inhibitors with a direct effect on specific targets in fungal cells or the improvement of existing drugs. Stress signaling in fungi is increasingly being recognized as important in adaptation to drug pressure, and its components are therefore being identified as potential targets (5, 6). The inhibition of some fungal cascades may be effective for both direct fungal and indirect synergistic approaches.

Ambient pH is one of the extracellular stresses to which these microorganisms must adapt rapidly. The major human pathogenic fungi, *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*, can survive in several anatomically distinct sites and can, therefore, provoke deep-seated infections in susceptible patients. In these settings, adaptation to pH is crucial and enables human pathogens to invade the bloodstream and tissues and to cause disseminated infections (7). Ambient pH is thought to be stable in the human body, and somewhat alkaline (around 7.4), but extreme variations occur. For example, spatial changes are observed along the digestive tract, together with temporal changes in the vaginal cavity with changes in hormone pressure, and the skin is acidic. PacC/Rim signaling is the best-studied pH response pathway. It has been extensively described in model organisms such as *Aspergillus nidulans*, *Saccharomyces cerevisiae*, and *Yarrowia lipolytica* (8–17). In pathogenic fungi, this pathway elicits the primary pH response and has been shown to be required not only for survival and growth in the host but also for invasive progression in tissues and for virulence (7, 18).

In this review, we focus on the way in which the PacC/Rim pathway is processed in the main fungal species, highlighting both conserved and divergent mechanisms. The role of this pathway in virulence, pathogenesis, and modulation of the host immune response is also discussed, together with its involvement in adaptation to drug pressure, all of which make this fungus-specific system a promising target for innovative antifungal strategies.

pH SIGNAL PROCESSING IN FUNGI

Fungi can grow at a wide range of pH values. The signaling pathways mediating responses to pH therefore play a key role in the cell biology of these organisms. Neutral-alkaline sensing relies mainly on the pathway called PacC in filamentous fungi and Rim101 in yeasts (7, 13–15, 18–21). This pathway is functional in deuteromycetes, ascomycetes, and basidiomycetes and is well conserved,

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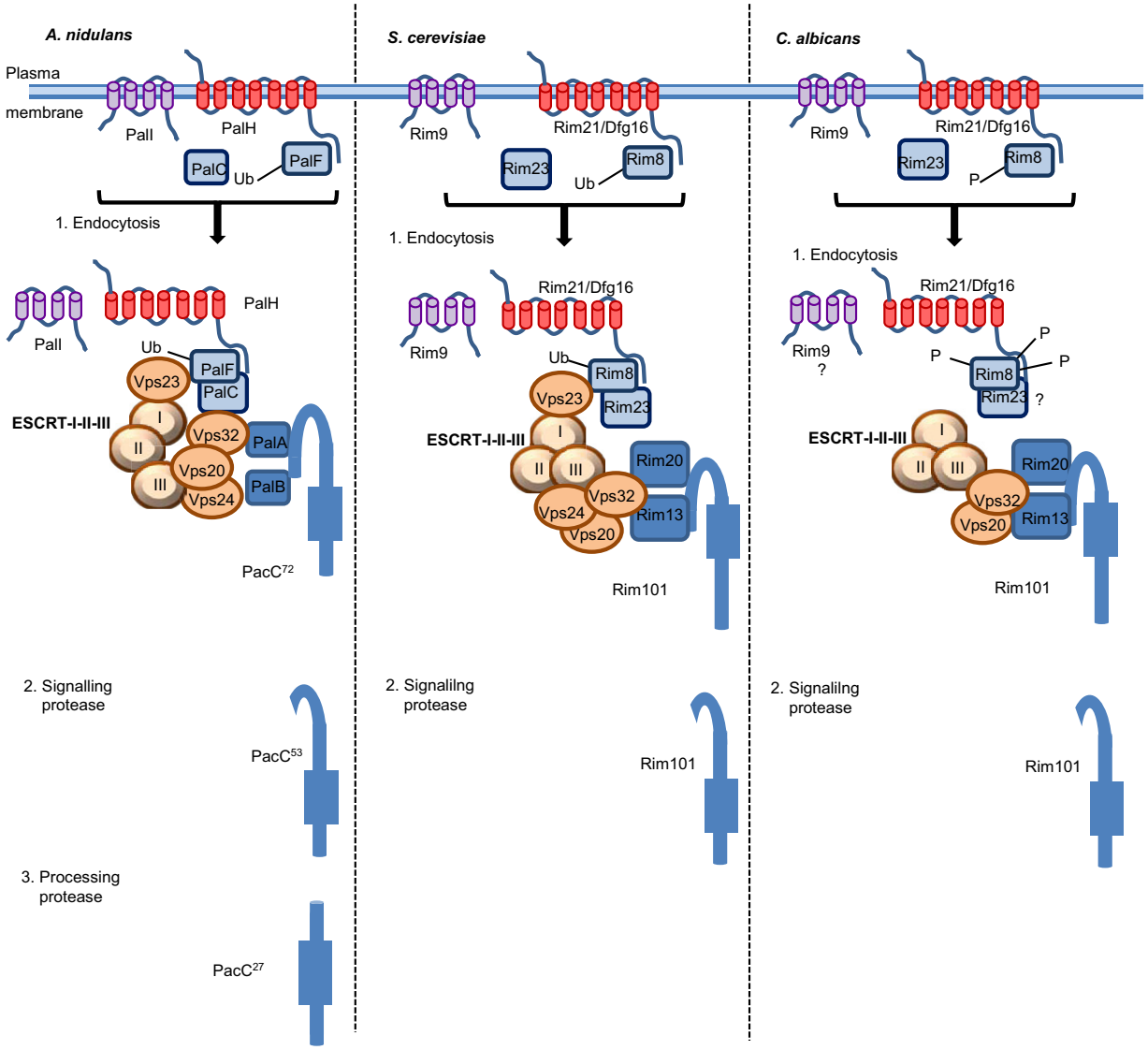

FIG 1 Pal/Rim signaling cascades in *Aspergillus nidulans*, *Saccharomyces cerevisiae*, and *Candida albicans*. External pH sensing involves the plasma membrane complex, comprising PalH/Rim21-Dfg16 and its assistant, PalI/Rim9, an arrestin-like protein PalF/Rim8, and PalC/Rim23. At alkaline-neutral pH, the plasma membrane complex is endocytosed through PalF/Rim8 ubiquitination, which triggers interactions with the ESCRT complexes involved in the multivesicular body endocytic pathway. Vps32, a key component of ESCRT-III, interacts with PalC/Rim23 and PalA/Rim20. PalA/Rim20 in conjunction with PalB/Rim13 (the signaling protease) are members of the endosomal signaling complex and recruit PacC/Rim101. PalB/Rim13 mediates the cytoplasmic proteolysis of the full-length PacC/Rim101. In *A. nidulans*, a second processing event occurs independently of PalB. In all species, whether PalI/Rim9 is endocytosed together with PalC/Rim23, PalH/Rim21, and PalF/Rim8 has not been demonstrated. In *C. albicans*, the role of Vps23 has not been described. Also in *C. albicans*, Rim8 is not ubiquitinated but hyperphosphorylated and it associates with Rim21 and Rim101, suggesting a role in bridging the cortical and the endosomal signaling complexes.

although marked differences have been noted between these three groups of fungi (20, 22, 23). The regulation of pH has been studied most extensively in the nonpathogenic species *A. nidulans*, but studies of the yeasts *Saccharomyces cerevisiae* and *Yarrowia lipolytica* have also contributed to our current understanding. Thus, after a brief description of pH signal transduction and its multiple sequential steps in model organisms, we will focus on the situation in pathogenic fungi. We will concentrate on recent advances and refer to reviews for a comprehensive analysis of previous data (7, 13, 14, 24).

PacC PROCESSING IN *ASPERGILLUS NIDULANS*

There are seven dedicated proteins involved in the PacC signaling pathway: PacC, the transcription factor, PalH, PalI, PalF, PalC, PalA, and PalB, which relay changes in environmental pH to PacC (Fig. 1). External pH sensing involves a cortical membrane complex of three proteins: the putative pH sensor PalH and its assistant PalI, together with an arrestin-like protein PalF. PalF binds to the C terminus of PalH and becomes phosphorylated and ubiquitinated at neutral to alkaline pH in a PalH-dependent manner (25–28). The N-terminal arrestin domain of PalF has been shown

to be crucial for the PalF-PalH interaction and further activation of this pathway. This domain is also highly conserved in pathogenic fungi, such as *A. fumigatus* and yeasts (25, 28). Ubiquitinated PalF then binds the Bro1 domains of PalC and Vps23, a component of endosomal-sorting complex I required for transport (ESCRT-I) (27, 29, 30). This triggers the sequential recruitment, to the cortical region located at the plasma membrane, of additional Vps proteins that are normally part of the ESCRT complexes of the multivesicular body of the endosome, together with the two remaining Pal proteins (PalA and PalB). The Vps32/Vps20 subcomplex, a key component of ESCRT-III, interacts with PalC and PalA, whereas Vps24 recruits and possibly activates PalB, the signaling protease (29, 31, 32). In response to neutral to alkaline conditions, PalB mediates the cytoplasmic proteolysis of the full-length 72-kDa $PacC^{72}$ precursor, generating the N-terminal 53-kDa fragment $PacC^{53}$ (33, 34). $PacC^{53}$ is the substrate of a second, possibly proteasomal, proteolytic event yielding the 27-kDa final product, $PacC^{27}$. $PacC^{27}$ is then translocated to the nucleus, where it turns on genes responsive to alkaline conditions and switches off genes responsive to acidic conditions (35). In summary, the current model suggests that the PacC pathway is triggered at alkaline pH by cortical structures at the plasma membrane that recruit all Pal proteins and components of the ESCRT endosomal machinery for the activation of PacC by proteolysis (Fig. 1 and Table 1) (30).

In *Saccharomyces cerevisiae* and *Yarrowia lipolytica*, the PacC/Rim101 pH transduction pathway is called Rim, because *RIM101* was first identified as a regulator of *IME2*, a gene involved in meiosis initiation in *S. cerevisiae*. This pathway was subsequently studied mostly for its role in the pH response mediated by Rim101 (formerly Rim1, the PacC orthologue in yeasts) (12, 36–38). All components of the *A. nidulans* PacC pathway have homologues in yeasts. However, the selection of yeast mutants with impaired pH responses and the identification of proteins interacting with known Rim proteins led to the identification of additional players, mostly components of the endocytic pathway that were later shown to have similar, but not identical, functions in *A. nidulans*. Interestingly, all single *vps* mutants are viable in *S. cerevisiae*, but *vps32* mutants are lethal in *Y. lipolytica*, and all *vps* mutants are lethal in *A. nidulans* (8–10, 16, 17, 29, 32, 39–42).

One of the major differences between PacC and Rim101 pathways is that only one proteolytic event, corresponding to the step generating $PacC^{53}$ in *A. nidulans*, has been demonstrated in *S. cerevisiae* and is thought to occur in other hemiascomycetous yeasts at alkaline pH. The processed form of Rim101 is thus analogous to the $PacC^{53}$ form (12, 16, 36) (Fig. 1).

Another difference concerns the PalH pH sensor, which exists in *S. cerevisiae* as two paralogues, Dfg16 and Rim21, thought to form a heterodimeric receptor (Table 1). Both have been shown to be required for signal transduction (43, 44). Recent results demonstrated that the PalI homologue Rim9 forms a complex with Dfg16p and Rim21p that is localized at the plasma membrane and that these three factors are mutually dependent, with Rim21 the most important and Rim9 and Dfg16 playing secondary roles by maintaining Rim21 levels and guiding the membrane localization of this protein (45).

It was long thought that yeasts had no *PALC* homologue. *YGR122*w (called *RIM23*) has recently been identified as a putative *PALC* homologue in *S. cerevisiae*, suggesting that the entire pathway may be conserved in hemiascomycetous yeasts (44). Like PalC in *A. nidulans*, Rim23 has been shown to be required for Rim101 activation in *S. cerevisiae* and *Y. lipolytica* (Fig. 1 and Table 1) (8, 10, 29). In *Y. lipolytica*, Rim23p also assembles the early ESCRT components Vps27 and Vps23, and *vps23* mutants have been shown to have a Rim phenotype (8). Identical domains of Vps23 bind Rim8 (the PalF orthologue) in the pH-sensing Rim pathway and Vps27 in the endocytosis-specific ESCRT pathway, suggesting that Vps23 acts at the crossroads of pH signaling and endocytosis (8).

In *S. cerevisiae*, interaction between Vps23 and Rim8 is favored by the ubiquitination of Rim8, but these two events have been shown to occur independently of ambient pH and are not critical for Rim101 processing (46). The differential pH dependence of the ubiquitination of Rim8/PalF and its interaction with Vps23 is another distinction between *S. cerevisiae* and *A. nidulans* (Table 1). According to the current model (Fig. 1), the ubiquitinated Rim8-Vps23 complex binds to Rim21, leading to the recruitment of Rim23. Rim23 then associates the ESCRT machinery with the plasma membrane complex (27, 46). Furthermore, coimmunoprecipitation studies with *S. cerevisiae* have indicated that the Vps20/Vps32 heterodimer forms a complex with Vps24, Rim13 (the PalB orthologue), and Rim20 (the PalA orthologue) and that the binding of Rim13 and Rim20 is not affected by *vps32* mutations that affect Vps24p binding (47–50). Thus, the recruitment of Rim13 to the endosomal processing complex may differ between yeasts and *A. nidulans*.

SPECIFIC FEATURES OF Rim101 PROCESSING IN *CANDIDA ALBICANS*

In *C. albicans*, the single proteolytic event that mediates Rim101 activation at alkaline pH requires the same protein signaling cascade as in *S. cerevisiae*. External pH sensing involves Rim21, Dfg16, Rim9, and Rim8, and we and others have shown that the ESCRT-I, -II, and -III endosomal complexes play conserved roles in pH signal transduction in *C. albicans* (17, 39–42). However, *C. albicans* differs considerably from *A. nidulans* and *S. cerevisiae* in terms of the signaling step, the role of the cortical membrane structure, and its link to the Rim101 proteolytic event (Table 1).

In *C. albicans*, it has been shown that Rim8 is sequentially post-translationally phosphorylated rather than being ubiquitinated, and hyperphosphorylation is linked to Rim101 processing, occurring at the same time or after this processing. The ESCRT factors required for Rim101 activation are also required for Rim8 phosphorylation. It has also recently been shown that, under alkaline conditions, Rim8 associates with both Rim21 and Rim101, suggesting that Rim8 may be involved in the link between the cortical signaling complex and the Rim101 proteolysis step (Fig. 1) (51).

Another important key feature specific to the *C. albicans* pathway is the presence of a negative feedback regulatory loop involving both Rim101-mediated repression and the degradation of Rim8 in the vacuole, leading to a decrease in protein levels at high pH (51–53) (Table 1). The ESCRT factors involved in Rim101 activation are also required for Rim8 phosphorylation, which leads to the association of Vps32 and other Vps factors with the endosomal membrane complex. Vps32 is then able to recruit Rim20, Rim101, and Rim13, leading to the cleavage of Rim101 (9, 17, 32, 51, 54) (Fig. 1). As reported for *S. cerevisiae*, in which the deletion of *RIM9*, the PalI homologue, prevents Rim101 cleavage, we have shown that Rim9 is fully required for pH signal transduction in *C. albicans*, whereas *rim9* mutants have a leaky phenotype

TABLE 1 pH signal processing in fungal model organisms and in the main human fungal pathogens

Processing step	Fungal species				
	Aspergillus nidulans	*Saccharomyces cerevisiae*	*Candida albicans*	*Aspergillus fumigatus*	*Cryptococcus neoformans*
Sensing					
Cell location	Plasma membrane	Plasma membrane	Plasma membrane	Plasma membrane	Not described
Proteins (orthologues)	PalH with 7 TMDs,[a] PalI with 4 TMDs, PalF arrestin like	Dfg16/Rim21(PalH), Rim9 (PalI), Rim8 (PalF)	Dfg16/Rim21, Rim9, Rim8	PalH, PalF	Not described
Trigger	PalH-PalF interaction, PalF phosphorylation and ubiquitination (neutral-alkaline pH dependent)	Rim21-Rim8 interaction, Rim8 phosphorylation and ubiquitination (pH independent)	Rim21-Rim8 interaction, Rim8 hyperphosphorylation dependent on Rim101 activation	Not described[b]	Not described
Signaling					
Cell location	Plasma membrane, endosome	Plasma membrane, endosome	Plasma membrane, endosome	Not described[b]	Not described
Proteins (orthologues)	PalH, PalF, PalC, Vps23 (ESCRT-I[c]), Vps32/Vps20, Vps24 (ESCRT-III[c])	Rim21, Rim8, Rim23 (PalC), Vps23, Vps32/Vps20, Vps24	Rim21, Rim8, Rim23, Vps32/Vps20	Not described[b]	Not described
Trigger	Binding of ubiquitinated PalF-PalH to PalC and Vps23, PalF-dependent endocytosis of PalH through Vps23 recruitment of Vps32/Vps20	Binding of ubiquitinated Rim8-Rim21 to Rim23 and Vps23, Rim8-dependent endocytosis of Rim21 through Vps23 recruitment of Vps32/Vps20/Vps24	Interaction of hyperphosphorylated Rim8 with Rim21and Rim101, Rim8-dependent endocytosis of Rim21 through Vps32/Vps20 binding	Not described	Not described
Processing of PacC/Rim101					
Cell location	First proteolysis in endosome, second proteolysis in proteasome	Single proteolysis in endosome	Single proteolysis in endosome	Not described[b]	Not described
Proteins	Interaction of Vps32/Vps20 with PalA, and Vps24 with PalB. First PalB-mediated proteolysis of PacC[72] to generate PacC[53]. Second proteolysis mediated by the proteasome	Interaction of Vps32/Vps20/ Vps24 with Rim20, Rim13, and Rim101, Rim13-mediated proteolysis of Rim101	Interaction of Vps32 with Rim20, Rim13, and Rim101, Rim13-mediated proteolysis of Rim101 to generate Rim101[65]	Not described[b]	Not described, but two proteolysis steps dependent on Rim13, Rim20, and cAMP/PKA pathway
Trigger	PacC[27] translocation to the nucleus	Translocation to nucleus of Rim101 truncated form corresponding to PacC[53]	Translocation to the nucleus of Rim101[65]	Not described	Not described
Activation					
Cell location	Nucleus	Nucleus	Nucleus	Not described	Not described
Proteins	Binding of PacC[27] to pH responsive genes	Binding of truncated Rim101to pH-responsive genes	Binding of truncated Rim101to pH-responsive genes	Not described	Not described
Regulation					
Proteins	PacC	Not described	Rim8, Rim101	Not described	Not described
Trigger	Autogenous PacC mediated activation	Not described	Rim101-mediated repression and degradation of Rim8 and autogenous Rim101-mediated activation	Not described	Not described

[a] TMD, transmembrane domain.
[b] Thought to be highly similar to that in *A. nidulans*.
[c] Endosomal sorting complex required for transport.

in *A. nidulans* and *Y. lipolytica* (36, 55). Our results also suggest that other factors may be required to bridge the plasma membrane and the endosomal membrane complexes of the Rim pathway and that Rim23 may also be involved in this link in *C. albicans* (55). A likely candidate for *RIM23* in *C. albicans* is orf19.2914, which encodes a protein carrying a Bro1 domain, like Ygr122w in *S. cerevisiae* and other PalC orthologues (29).

By contrast to what has been reported for *A. nidulans* and other

yeasts, some authors have observed the Rim13-dependent C-terminal cleavage of Rim101 under acidic conditions in *C. albicans*, which yields a 65-kDa truncated form. It has been suggested that this form regulates expression of pH-independent genes, involved, for example, in lithium or hygromycin resistance, but which are nevertheless affected by *rim101* mutations (56).

SPECIFIC FEATURES OF PacC PROCESSING IN *ASPERGILLUS FUMIGATUS*

In the major mold pathogen of humans, *A. fumigatus*, PacC null mutants are abnormally sensitive to alkaline culture media *in vitro* and display aberrant regulation of zinc homeostasis, an essential component of *A. fumigatus* virulence in murine hosts (57, 58). Electromobility shift analyses have shown that PacC proteolysis resembles the proteolysis observed in *A. nidulans* in terms of pH sensitivity and proteolytic cleavage sites (M. Bertuzzi et al., personal communication).

The PacC/Rim101 pathway has also been studied in other *Aspergillus* species that are usually considered saprophytic. *Aspergillus niger* is an exceptionally efficient producer of organic acids, resulting in the rapid acidification of its environment. This secretion of acids is thought to help this saprophytic fungus to degrade plant cell walls to obtain nutrients. This species can grow at pH values from 2 to above 8, and this has led to pH regulation and functional genomics studies, resulting in the identification of genes for all steps of the PacC/Rim101 pathway (19, 59, 60). Similarly, in *Aspergillus oryzae*, a homologue of the *A. nidulans* PalH has been characterized and shown to be required for pH sensing (61).

SPECIFIC FEATURES OF Rim101 PROCESSING IN *CRYPTOCOCCUS NEOFORMANS*

As a saprophytic soil fungus, *C. neoformans* can sense and respond to multiple stresses and changes in its environment. Adaptation to pH is critical for *C. neoformans* pathogenesis in humans, as this basidiomycete can grow well at physiological pH in the blood and cerebrospinal fluid (pH 7.4) but also at the acidic pH found in the vesicles of phagocytic cells, such as macrophages. Experiments both *in vitro* and *in vivo* have demonstrated that phagosomes containing *C. neoformans* undergo acidification soon after phagocytosis and that the fungus can thrive and multiply for subsequent extrusion in a viable form or exocytosis (62–64). Furthermore, capsule production, a key virulence factor in this fungus, is optimal at physiological neutral-alkaline pH (23). The Rim101 orthologue in *C. neoformans* has recently been identified and shown to have similar physiological functions to the Rim101/PacC proteins of other fungal species, including roles in pH adaptation, salt tolerance, and iron homeostasis/import.

Rim101 processing and cleavage at alkaline pH occur in two steps, as in *A. nidulans*, and they are not only triggered by the Rim pathway, but also dependent on the cyclic AMP (cAMP)/protein kinase A (PKA) pathway, in marked contrast to the situation in ascomycetous fungi or yeasts (Table 1). Unlike its ascomycete homologues, Rim101 actually harbors a functional PKA consensus phosphorylation site. Rim101 is located in the nucleus regardless of pH, whereas in other fungi, PacC/Rim101 is mostly cytosolic under acidic conditions, with nuclear localization requiring both Rim13- and Rim20-dependent cleavage of Rim101 and an active PKA pathway (23).

INVOLVEMENT OF pH SIGNALING IN FUNGAL PATHOGENICITY

PacC/Rim101 effectors and downstream targets involved in pathogenesis. The genes and activities controlled by PacC/Rim101 in the model organisms *A. nidulans*, *S. cerevisiae*, and *Y. lipolytica* can be classified into several functional categories, including siderophore biosynthesis and iron or copper metabolism, ion homeostasis, the production of extracellular alkaline or acidic proteases or permeases, the production of enzymes involved in the synthesis of exported metabolites, membrane or cell wall biosynthesis and remodeling, sporulation, mating, dimorphism, invasive growth, and biofilm formation (12–15, 65–69). In pathogenic fungi, most of these functions are related to pathogenesis and virulence.

pH signaling and *Candida albicans* virulence. A comparison of the multiple steps of PacC/Rim101 processing between model and human pathogenic fungi (see above and Table 1) shows that the pathway is more refined in *C. albicans* than in the model organisms with a specific and accurate negative feedback control system (51). No such negative feedback has yet been described in other species, and its relevance to pathogenicity has not been demonstrated, but this regulatory mechanism may be associated with the higher levels of pH stress with which this commensal organism must deal to survive and to invade various cells or niches of the human body.

The main contribution of the Rim pathway to *C. albicans* virulence relates to its role in adaptation to neutral-alkaline conditions, through growth, iron transport and metabolism, pH-dependent morphogenesis, cell wall structure, adhesion, and the ability to produce biofilms (7, 68, 70–73). Impaired fungal growth under alkaline conditions results partly from the lower solubility of the ferric ions (Fe^{3+}), leading to iron starvation. One of the principal Rim101-dependent responses to alkaline conditions involves adaptation to iron starvation. Tolerance of iron deprivation at physiological pH is one of the most important virulence determinants of fungi, as freely available iron is strongly limited in human hosts, protecting the host against microorganisms unable to increase iron uptake (7, 52, 68, 74–78). Transcriptional analyses have identified several genes involved in iron metabolism and transport, including some Rim101-dependent targets, such as the siderophore iron transporter genes *ARN1* and *FET3* and the ferric reductase and ion permease genes *ENA1*, *RBT2*, *FRE2*, *FRE5*, *FRE8*, *FRP1*, *FRP2*, *CTR1*, and *ZRT1* (52, 74, 79). *ALS3* also is dependent on Rim101. In addition to its role in adhesion, Als3 binds to human ferritin, facilitating iron acquisition by *C. albicans* under host conditions (79, 80).

Both yeasts and hyphae can invade tissues, but the ability to shift from the yeast form to the hyphal form has been clearly linked to virulence (73, 81–83). Mutants unable to undergo morphogenic switching display attenuated virulence (39, 84). According to the pathophysiological model of invasive candidiasis in humans, hyphal forms are better able to adhere and to maintain the colonization of mucosal niches, to disrupt the epithelia of both mucosal and endothelial surfaces and, thus, to disseminate into the bloodstream and to invade deep-seated tissues (73, 82, 83). The yeast-to-hypha transition is also involved in interactions between fungi and bacteria and in the construction of biofilms, which play an important role in the pathogenicity of *C. albicans* (85–88). Rim101 upregulates filamentation, and many of the

genes strongly regulated by Rim101 are hypha-specific genes (*ECE1*, *CSA1*, *CSA2*, *SAP5*, *HYR1*, *HWP1*, *RBT1*, and *IHD1*) (52, 70, 73, 79). Genes involved in cell wall structure and remodeling (*ALS3*, *PGA7/RBT6*, *CHT2*, *SKN1*, *PHR1*, and *PHR2*) (18, 68, 79, 89) are also Rim regulated and make a major contribution to Rim-dependent pathogenesis, as many determinant factors for virulence are dependent on the cell wall (reviewed in references 81 and 90). Phr1 and Phr2 are paralogues that are differentially expressed under alkaline-neutral and acidic conditions, respectively. They are β(1-3)glucanosyltranferases involved in glucan remodeling of the cell wall (91, 92). Als3, Pga7/Rbt6, Hwp1, and Sap5 are adhesins that play a key role in invasion and biofilm formation (79, 81, 83, 93). Thus, the main contribution of the PacC/Rim101 pathway to *C. albicans* virulence is related to its role in adaptation to neutral-alkaline conditions, through iron homeostasis, pH-dependent morphogenesis, cell wall synthesis, and remodeling (7, 18, 68, 71, 94).

Experimental studies in both murine and porcine models have established the key role of the Rim101 pathway in *C. albicans* pathogenesis in disseminated candidiasis, keratitis, and oropharyngeal and intra-abdominal candidiasis (71, 79, 95, 96). The importance of pH regulation during tissue invasion through hypha formation and pathogen survival has been demonstrated in *in vivo* and *ex vivo* genome-wide transcriptional profiling comparisons for liver invasion. *ALS3*, *PHR1*, *ECE1*, *HWP1*, and *DFG16* were shown to be upregulated during liver invasion (93). Also, in a model of intra-abdominal candidiasis, *RIM101* was identified as one of the most strongly expressed genes, and a *rim101* mutant was found to cause a less-pronounced infiltration of the peritoneal fluid with neutrophils (97).

pH SIGNALING AND *ASPERGILLUS FUMIGATUS* VIRULENCE

After initial studies demonstrated the role of Rim-mediated pH signaling in *C. albicans* virulence, further analyses made use of the multiple *A. nidulans* pH mutants available, together with a neutropenic model of pulmonary aspergillosis to dissect the roles of PacC, PacC processing, and Pal-mediated pH signaling in *Aspergillus* spp. virulence (98). PacC action was found to be both required for and able to enhance virulence. The *A. nidulans* pH-responsive transcription factor PacC thus plays a key role in pulmonary pathogenesis. The PalH homologue was recently shown to be required for murine infection (99).

Transcriptome analyses comparing *in vitro* responses to alkaline stress and gene expression during the initiation of murine infection recently identified *A. fumigatus* adaptation to alkaline stress as a key component in the early stages of mouse infection (100). Consistent with these findings, the virulence of *A. fumigatus* PacC null mutants is attenuated in murine models of pulmonary aspergillosis. In addition to zinc homeostasis, many other virulence-enabling functions are also under the control of PacC regulation during *A. fumigatus* infection. This suggests that in *A. fumigatus*, PacC acts as a master regulator of multiple virulence factors and that the abolition of PacC-mediated pH signaling can greatly decrease virulence (M. Bertuzzi et al., personal communication).

pH SIGNALING AND *CRYPTOCOCCUS NEOFORMANS* VIRULENCE

Several virulence factors have been identified in *C. neoformans*, but the capsule is probably the major one (101). This complex structure is composed mainly of polysaccharides that are linked to the cell wall (102). Mutant strains unable to synthesize a capsule are avirulent, and the capsular polysaccharides exert profound depressive effects on both innate and adaptive immune responses (103, 104). Different factors, including pH, have been shown to influence the capsule size and structure (105). Rim101 plays a role in capsule formation in association with the cAMP/PKA pathway, probably by facilitating capsule anchorage to the cell wall rather than regulating capsule biosynthesis (23). Transcriptional profiling has identified several Rim101-dependent genes, including those encoding a UDP-glucose dehydrogenase, a manosyltranferase, and a phosphomannomutase, which are essential for capsule synthesis. However, many of the genes involved in capsule production are not dependent on Rim101. Other downstream targets of Rim101, such as *ENA1*, which is directly regulated by Rim101, are associated with the alkaline response or with iron or metal homeostasis (*CFT1*, *FET3*, and *SIT1*). As in other fungi, *rim101* mutants display impaired growth under alkaline or iron starvation conditions. Interestingly, Vps23 has been shown to be involved in iron acquisition, capsule formation, and virulence. However, these functions may be explained by the role of Vps23 in endocytosis, and its contribution, like other ESCRT factors, in Rim101 processing in *C. neoformans* has not been demonstrated yet (106). Many of the genes involved in cell wall biosynthesis and remodeling are regulated by Rim101 in *C. neoformans* (*CHS4*, *CHS5*, *CHS6*, *CHS8*, *SKN1*, *FKS1*, *KRE6*, *CDA3*, *CHI22*, and *AGS1*) (107). Rim101 is also required for the formation of titan cells, a type of enlarged yeast cell. Titan cells are more resistant than normal yeast cells to engulfment by immune cells, and they are also more resistant to oxidative and nitrosative stresses, providing normal cryptococcal cells with cross-protection (107–109). The role of the Rim pathway in this pathogenesis-related morphological switch in *C. neoformans* is reminiscent of the Rim-dependent yeast-to-hypha transition displayed by *C. albicans* in the host.

Despite these defects in the capsule, alkaline pH adaptation, iron homeostasis, and morphological switching, the *rim101*-deleted strain was found to be hypervirulent in a mouse model of disseminated cryptococcosis with inhalation as the route of inoculation (23, 110). These rather unexpected results were initially attributed to greater survival of the *rim101* mutant within macrophages due to the derepression of acid response genes, leading to increased survival under acidic conditions (23). However, it now also appears likely that the capsule attachment defect has a profound effect on the host immune response. First, enhanced diffusion of the capsule polysaccharides into the surrounding tissues may increase their immunomodulatory effects. Second, as the cell wall of the *rim101* mutant cells contained higher than normal levels of antigenic mannoproteins (MP88 and MP98), inappropriate recognition and responses may result in excessive host inflammation and, eventually, in cell death. Recent *in vivo* experiments have confirmed that there is an exacerbation of the neutrophil and cytokine defenses that results in a deleterious and ineffective inflammatory response (107). The capsule defect itself worsens this disproportionate immune response by unmasking the hyperimmunogenic cell wall. The hypervirulent phenotype displayed by the *rim101* mutant and the role of the pathway in altering the antigenic properties of the cell wall have not been demonstrated in other species, further highlighting the profound differences in the PacC/Rim101 pathway between ascomycetes and basidiomycetes. Further studies focusing on the implication of pH signaling in

alterations to host-pathogen interactions through cell wall remodeling are warranted (20, 22, 23, 110).

THE POSSIBLE USE OF pH SIGNALING AS A DRUG TARGET FOR ANTIFUNGAL STRATEGIES

Recent studies have highlighted the potential of the Pac/Rim pathway as a source of targets for improving antifungal strategies. The results detailed above highlight the role of this pathway in iron homeostasis, morphological switching under host conditions, cell wall synthesis, and remodeling. Together, they provide a basis for this hypothesis: targeting iron uptake by blocking siderophore biosynthesis, or through the use of anti-Als3 antibodies or chelators, has been shown to reduce *Aspergillus*, *Candida*, *Fusarium*, and *Zygomycetes* infections in animal models and in clinical trials (80, 111–115). Given its unique composition and its role in the host-pathogen interface in modulating both the immune response and morphogenetic switching, the cell wall remains an ideal target. However, its dynamic structure makes adaptation possible, and fungi are thus able to respond to drugs targeting the cell wall (116, 117). We summarize here additional studies from our group and others whose findings implicate the Pac/Rim pathway in the cellular responses underlying antifungal tolerance.

Tolerance to azoles and caspofungin was first described by Sanglard and coworkers, who established the role of the calcineurin pathway in such tolerance in *C. albicans* (118–120). The compensatory mechanisms involved in antifungal tolerance are mediated by a complex interplay between the calcineurin, Tor, HOG, and PKC-cAMP-PKA signaling pathways, resulting in an increase in chitin synthesis (120–127). Compromise of PKC signaling and the chaperone Hsp90 leads to similar killing effects as calcineurin inhibitors on ergosterol biosynthesis inhibitors and echinocandins (3, 124, 128–132). The mechanisms of antifungal drug tolerance in *A. fumigatus* are similar to those described for *C. albicans*, although they have been explored less thoroughly. The calcineurin blockade has been shown to enhance the inhibition of 1,3-β-D-glucan by caspofungin in *A. fumigatus* and to convert caspofungin into a fungicidal drug (133, 134). Hsp90 is also involved in the echinocandin tolerance of *A. fumigatus* and *Aspergillus terreus* (129, 135). In *C. neoformans* too, the combination of azoles or caspofungin with calcineurin inhibitors was found to be synergistic, but this may reflect the known ability of FK506 to block the pumps involved in multidrug resistance (136). As with *C. albicans*, mutants of the PKC pathway display enhanced susceptibility to caspofungin (137).

A role of pH regulation in antifungal activity was suggested by the observation that the azole compound D0870 had higher levels of fungicidal activity against *C. neoformans* at low pH (138). Similar results were subsequently obtained in susceptibility tests under acidic conditions, which were found to inhibit the trailing effect of the azoles in *Candida* spp. (139). This trait, which hampers interpretation of the activity of azoles and echinocandins against yeasts, is not fully understood, but it may relate to the lack of fungicidal activity of these drugs (which are instead fungistatic) (139, 140).

The role of the PacC/Rim101 pathway in azole tolerance has been explored in *S. cerevisiae*. In a chemical-genetic and genetic interactions study, all mutants of the pathway were found to be hypersensitive to fluconazole, and the double *rim20-erg11* mutant was not viable, with Erg11p the enzyme targeted by azoles (141). We previously showed that a *rim101*-deleted strain of *C. albicans* also displayed enhanced azole susceptibility. Deletions of *VPS28* and *VPS32*, encoding endocytic components of the endosomal membrane complex of the PacC/Rim101 pathway, resulted in the same phenotype of azole hypersusceptibility. The constitutively active truncated *RIM101SL* gene restores azole susceptibility in all *rim101* and *vps* mutant strains, demonstrating the involvement of the PacC/Rim101 pathway in *C. albicans* tolerance to azoles (142). The azole hypersensitivity phenotype was subsequently confirmed by others as part of a large phenotypic profiling experiment on a collection of strains with deletions of genes encoding transcriptional regulators (143). Furthermore, we recently confirmed that all *C. albicans rim* mutants, not just the *rim101* mutant, were hypersensitive to all the triazoles used to treat humans (fluconazole, voriconazole, and posaconazole) and that Rim disruption rendered these compounds fungicidal rather than fungistatic (144). In *A. fumigatus*, iron homeostasis has been linked to ergosterol synthesis and azole resistance (112). A recent study demonstrated a role for the PacC/Rim101 pathway in *C. neoformans* tolerance to azoles and amphotericin B through regulation of the cation transporters Ena1 and Nha1. As these transporters are involved in membrane stability and cation homeostasis, their inhibition enhances azole and polyene activity (145).

CONCLUSIONS

Many of the features described above support the hypothesis that the pH signaling pathways could be targeted in a novel approach to decrease fungal development and virulence in the infected host. We assume that blockade of the PacC/Rim101 pathway is likely to have beneficial and synergistic effects, by reducing the virulence and growth of pathogenic fungi and by decreasing the tolerance and resistance of these fungi to existing antifungal drugs. Furthermore, the specificity of the PacC/Rim101 pathway and its conservation throughout the fungal kingdom suggest that this pathway has considerable potential as a therapeutic target.

Defining the proper target(s) is of course an essential step. While multiple targets can be envisioned to inhibit the Pac/Rim pathway, targets that are both well conserved in pathogenic fungi and are fungus specific should be prioritized (25, 28, 99, 146). Fungus-specific components of the Pac/Rim pathways in pathogenic yeasts and fungi need to be fully characterized, both from a genetic and a biochemical point of view, in terms of mutant phenotype, protein structure, subcellular localization, and interactions. The molecular mechanism of pH sensing remains itself mysterious, and definite proof of the role of the alleged sensors is still missing. Interactions between the pH-sensing pathway and mechanisms of fungal tolerance represent another area poorly explored up to now. Genetic screens for chemical-genetic interactions (141) of Pac/Rim mutants with antifungal drugs may shed light on this aspect. These experiments will identify targets for adjunctive therapies to existing drugs in order to obtain fungicidal rather than fungistatic activity or to lower fungal tolerance to existing drugs, thus decreasing emergence of resistance.

Finally, it has become apparent that the fungal pH signaling pathways, while being highly conserved in terms of components, are entangled in highly diversified genetic networks in different organisms. These remain to be precisely deciphered to appreciate fully the range of variations that can be expected. Further studies of the pH signaling pathway in different pathogenic fungi may thus answer some of these questions and hopefully help to design new drugs badly needed in both medicine and agriculture.

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